STUDIES ON THE PHYSIOLOGY OF LIGNIN DECOMPOSITION BY SOIL FUNGI

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ONE of the most important problems in the decomposition of organic matter under natural conditions is the question of the breakdown of lignin. However, investigations concerning lignin are beset with difficulties, the principal one being that we do not know, from the chemical point of view, exactly what lignin is. Brauns, in his excellent book (1952) remarks, 'we do not even know the exact structure of the building stones (of lignin), which was known for cellulose 100 years before its structure was finally established'.

Chemistry of lignin and its occurrence in plant materials and in soils

Present-day indications are that lignin is a complex polymer built up from certain basic units. Also it is recognized that different kinds of lignin vary in their basic units. Lignin is known to contain carbon, hydrogen, and oxygen (Brauns, 1954). It is also known to contain methoxyl and hydroxyl groups, to have an aromatic structure, and probably a propyl side chain on at least some of the aromatic rings. Several theories for lignin structure are based on a phenyl-propane building unit with methoxyl and hydroxyl groups attached to it, e.g. the guaiacyl-propane unit (Fig. 1), but it is not yet certain how the units are

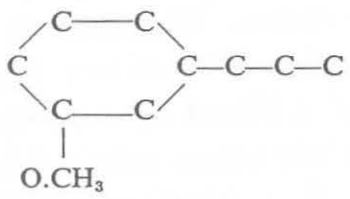


Fig. 1. Guaiacyl-propane unit.

attached to one another. According to Freudenberg (Brauns, 1954), the units condense with the formation of benzofuran and benzopyran rings which are linked in chains, while Erdtman (1949) states that the units are linked in chains with carbon-carbon and carbon-oxygen-carbon linkages. Certain units can be released on alkaline nitrobenzene oxidation of lignin—thus vanillin is derived from softwood lignins, syringal-

dehyde and vanillin from hardwood lignins, and *p*-hydroxybenzaldehyde from monocotyledon lignins (Creighton *et al.*, 1944). More recently, Leopold (1952) has shown that coniferous lignins may, in fact, also yield *p*-hydroxybenzaldehyde and syringaldehyde, in addition to vanillin which is the principal product.

Nor is it known how lignin occurs in plants, whether it is present in a mixture with the other constitutents or whether it is chemically combined with them. Lignin cannot be isolated from plant material in any great quantity. The only method by which unaltered lignin may be extracted is that of Brauns (1939) using alcohol. However, since this method yields only 8–10% of the total lignin content of wood, it would seem that the product is not representative of the lignin content of the plant as a whole. Other methods of extraction using acid and alkali give higher yields but are rather drastic, and the lignin is considerably altered during the extraction process.

Furthermore, it is not known in what form lignin occurs in the soil. Since it is one of the principal plant constituents, it is obvious that large amounts of it must be added to soil on the decay of plant material and, since it is much more resistant to biological decomposition than are the other plant constituents, it must accumulate in quantity and become a major component of soil organic matter. Evidence that lignin-like material does accumulate has been obtained by using the methoxyl content of soil and its solubility in 72% (v/v) H_2SO_4 as a measurement. The presence of typical lignin constituents in humic acid has been demonstrated, but according to Gottlieb & Hendricks (1945), it would seem that considerable alteration in the ratio of the different groups and their arrangement occurs. Waksman & Iyer (1932) believe that in soil lignin forms a complex with protein, and Hobson & Page (1932) claim that the nitrogen of artificial ligno-protein complexes acted similarly to humus nitrogen, while others refute this idea. However, work recently carried out by Dr. R. I. Morrison (1958) in the Biochemistry Dept. at the Macaulay Institute provides strong evidence for the occurrence in humus of a fraction derived directly from plant residues. On the alkaline nitrobenzene oxidation of soils and peats, the presence of syringyl, guaiacyl, and *p*-hydroxyphenyl residues was demonstrated, and the relative proportions of these groups were similar to those in the parent plant material, where this was known.

Microbiological decomposition of lignin

Attempts to study lignin decomposition by micro-organisms have been made using plant materials and preparations isolated by various

methods. However, little or no conclusive evidence has been obtained, partly due to the fact that pure, unaltered lignin cannot be isolated and partly to the absence of a reliable method for lignin estimation. Using plant materials, the indication from proximate methods of analysis, e.g. Waksman's (1926), is that lignin is decomposed by some fungi and bacteria, generally more slowly than are other plant constituents. Wood-rotting fungi have been divided into white-rots and brown-rots—the former attack lignin, but will also decompose cellulose; while the latter attack cellulose exclusively. Various isolated lignins have also been used, such as alkali, sulphuric acid, and phenol lignins; but results obtained with them are inconclusive—partly on account of their altered structure and partly on the presence as impurities of substances which can be utilized for growth by bacteria and fungi. In a few instances compounds related to lignin have been used. Konetzka *et al.* (1952) showed that α-conidendrin, which is structurally related to lignin, being composed of two guaiacyl-propane units, was attacked by *Flavobacterium* sp. Extracellular enzymes from *Polystictus versicolor* formed coloured products from conidendrin, syringaldehyde, vanillin, and ferulic acid. Dion (1952) attributed this to quinone formation by phenoloxidase activity which did not involve decomposition of the molecules.

The investigations of the author on lignin decomposition have been based on studies of the metabolism by fungi of some of the lignin-related molecules which are known to be released chemically from lignin and which are obtainable in pure form, e.g. vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde. They were supplemented by the study of the release of such units by fungi from untreated, naturally occurring material in the form of wood saw-dusts.

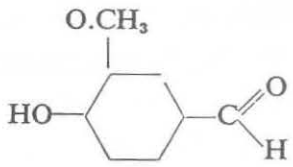
Isolation of possible lignin-decomposing fungi from soils

The fungi employed were isolated by Waksman's (1916) dilution-plate technique. The different areas selected for collection of soil samples were chosen on account of the plant community they supported, and isolates were obtained from broad-leaved and coniferous forests, a pasture, garden, heath, moor, peat moss, and sand dunes. Since the primary aim of the work was the collection of fungi concerned in the decomposition of lignin, a method selective for the isolation of such fungi was sought. Bavendamm (1928) and Davidson, Campbell & Blaisdell (1938) showed that wood-rotting fungi could be differentiated by their reaction with tannic acid. They found that white-rots, which are most active in decreasing the lignin content of wood, could oxidize tannic acid to a brown product, and when the acid was incorporated in

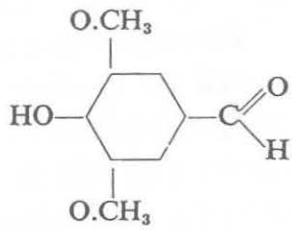
the medium the oxidation product was visible as a halo surrounding the fungal colony. It was hoped that lignin decomposers in soil might be selected in a similar manner, and 0·1% (w/v) tannic acid was always added to the modified form of Waksman's medium used for the dilutions. The medium was modified by the replacement of peptone by $(NH_4)_2SO_4$ to reduce the rate of growth of rapidly spreading fungi. Representative isolates from colonies not producing a brown halo as well as from those which did were cultured. A large number of species was obtained, but those which were identified all belonged to the Fungi Imperfecti.

Growth of soil micro-fungi on lignin-related molecules

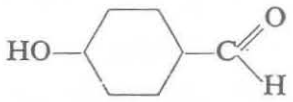
The lignin-related compounds used in the initial stages of the work (Henderson & Farmer, 1955) were vanillin, syringaldehyde, *p*-hydroxybenzaldehyde and ferulic acid (Fig. 2). The three former were selected since they are obtained on



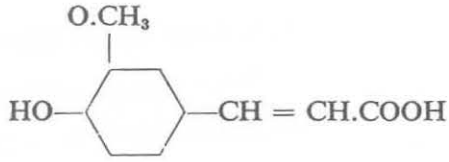
Vanillin



Syringaldehyde



p-Hydroxybenzaldehyde



Ferulic acid

Fig. 2.

alkaline nitrobenzene oxidation of various lignins, the latter as it contains the phenyl-propane unit regarded as being the basal unit of the lignin molecule. It has also been isolated from lignin (Smith, 1955). These compounds were added to 10 ml. of a mineral salts medium in a petri dish as sole source of carbon. The medium was Czapek's with sucrose omitted and since $FeSO_4$ reacts with phenols to form coloured compounds it was also omitted. The phenolic compounds are toxic to the fungi at about 0·04% (w/v), and to keep below this level the three aldehydes were present in a final concentration of 0·01% (w/v) and

ferulic acid at 0·006% (w/v). In this preliminary work about 60 different fungi were used. After 24 days incubation, the amounts of growth were noted, and although these were small there was definitely an indication that the phenols were supporting growth. The culture solutions were analysed by U.V. spectrometry, and the disappearance of the original phenols was estimated and in some cases intermediate metabolic products were identified. A close correlation was found to exist between the increased growth supported by the phenols and their disappearance as revealed by the spectrochemical analyses of the culture media at the end of the growth period. Certain fungi, e.g. *Alternaria* sp., *Hormodendrum* spp., *Penicillium* sp., and *Torula* spp. were more active than the others and caused complete disappearance of the ring structure of all four compounds, while other fungi attacked different compounds to different extents. As might be expected, the simplest substance, *p*-hydroxybenzaldehyde, was most readily decomposed. Vanillin was more quickly broken down than syringaldehyde. Generally decomposition of ferulic acid was similar to that of vanillin.

Intermediates identified in the decomposition of the phenols were vanillic acid, formed from vanillin and ferulic acid, and syringic acid, formed from syringaldehyde (Fig. 3). Some fungi seemed to be unable to metabolize the acids further and they accumulated in the media, but in many cultures no acid could be identified at all, indicating that it had been metabolized, while in others only small amounts or traces of acid were present. No further phenolic intermediates could be identified, from which it was concluded that if they were formed they were metabolized very rapidly, or that the ring was being ruptured at this stage. The spectrochemical method of analysis gave no information about products formed after breaking of the ring. Two fungi (*H. cladosporioides* and *Penicillium* sp.) grew on α-conidendrin and decomposed it, but it was not possible to identify any intermediates or end-products.

The results obtained by the spectrochemical analyses were augmented by others from paper chromatography. For this 100-ml. lots of medium were used and extracted with ether, the extractions being made after periods of a few days. In this way it was possible to confirm the formation of acid intermediates by those fungi which removed all traces of phenolic compounds during the 24-day incubation period.

Metabolism of lignin-related molecules by soil fungi

Warburg technique using spore suspensions. To support the results obtained from the growth experiments, respiration experiments were carried out in the Warburg apparatus. In an attempt to surmount the

well-known difficulties encountered when carrying out respiration experiments with fungi (the high endogenous respiration and the preparation of uniform suspensions), spore suspensions were used (Henderson, 1956). These were prepared by placing strips of cellophane bearing sporing growths of fungi in a solution of 0·1% (v/v) 'Tween 80' in a boiling tube. The spores were readily obtained in suspension by shak-

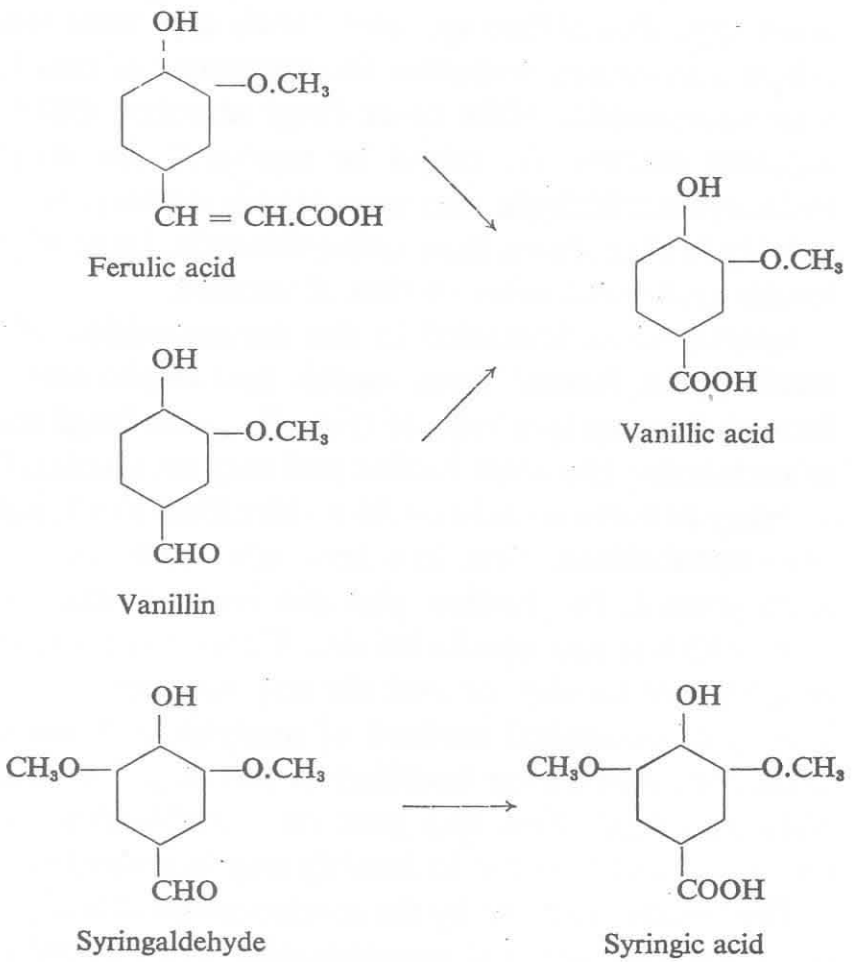


Fig. 3.

ing, and could be poured off, leaving the mycelium adhering to the cellophane. The spores were centrifuged down, washed twice with distilled water, and finally suspended in distilled water, a very uniform suspension being obtained, which could readily be pipetted into the Warburg flasks. In order to obtain sufficient activity, the suspension was incubated overnight in a mineral salts solution containing yeast extract. By morning germination had occurred and the added substrates had been utilized, so that the basal respiration rate was very low and marked increases in oxygen consumption were obtained on the addition of substrates. This method is necessarily restricted to fungi producing abun-

dant spores, but it was successfully used with species of *Hormodendrum*, *Haplographium*, *Penicillium*, and *Spicaria*. Since each experiment was continued over a period of about 24 hrs. altogether, it was essential to carry out all steps under aseptic conditions.

The oxygen uptakes obtained when the substrates were *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde, and vanillin could be correlated with the results obtained from the spectrochemical analyses of the culture solutions. *p*-hydroxybenzaldehyde, which was most readily utilized in the growth experiments, gave the most rapid oxygen uptakes. Syringaldehyde which was most slowly utilized gave the lowest oxygen uptakes, while ferulic acid and vanillin gave uptakes intermediate between the other two. The oxygen uptakes with the corresponding acids were also studied. In general, they were more slowly oxidized than were the aldehydes, which probably explains the tendency for them to accumulate in culture solutions. It was interesting to note that *p*-hydroxybenzoic acid was usually more rapidly oxidized than were the other two acids. It is the expected product from *p*-hydroxybenzaldehyde, but it was never traced in culture solutions, the indication being that it was further metabolized too rapidly for it to accumulate.

Kluyver experiments using preformed mats. Another technique was sought whereby conditions might be developed under which intermediates would accumulate in quantities sufficient for identification. For this purpose a modification of Kluyver & van Zijp's (1951) technique was used in which solutions of the experimental substances were poured under pre-grown fungal mats and incubated for various lengths of time. In this way it was possible to work with more concentrated solutions than could be used in the growth experiments. The culture solutions were extracted with ether as before, and the extracts were run on paper chromatograms. By this method *p*-hydroxybenzoic acid was readily obtained from *p*-hydroxybenzaldehyde, and protocatechuic acid was also identified. This indicated that the pathway of metabolism of *p*-hydroxybenzaldehyde was similar to that occurring in bacteria, according to which one would expect that breaking of the ring would take place after the formation of protocatechuic acid, with the subsequent formation of β-ketoadipic acid (Evans, 1947; Evans, Parr & Evans, 1949).

Recently (Henderson, unpublished), using trace element-deficient cultures of *Aspergillus niger*, it has been found that iron plays an important part at this stage. Dagley & Patel (1957) have already shown that protocatechuic acid oxidase in *Pseudomonas* sp. is dependent on ferrous ions, and we obtained confirmation of this when we found that

there was a definite accumulation of protocatechuic acid when iron-deficient cultures of *A. niger* were incubated over a solution of *p*-hydroxybenzoic acid. Also, with *o*-hydroxybenzoic acid and iron-deficient mycelium, an accumulation of catechol was obtained. Walker & Evans (1952) found that catechol is an intermediate in the breakdown of *o*-hydroxybenzoic acid by *Ps. fluorescens*, and, like protocatechuic acid, catechol is the product which immediately precedes rupture of the aromatic ring.

Since this technique was proving successful, it was also utilized in a study of demethoxylation (Henderson, 1957). It is a well-known fact that in soil a significant feature associated with the decomposition of lignin is the reduction in methoxyl content (Sowden & Atkinson, 1949).

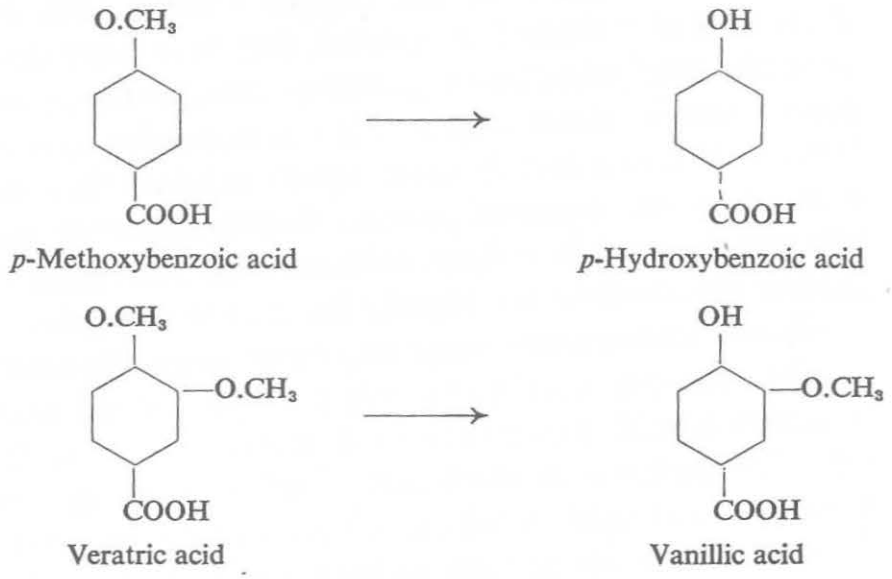


Fig. 4.

Mono- and di-methoxybenzoic acids were used for this work, and the decomposition of the acids was followed by taking samples from the flasks at intervals of a few days and analysing the samples by U.V. spectrometry. The formation of intermediates was studied by paper chromatography of ether extracts of culture solutions. The three mono-methoxybenzoic acids were all attacked by *Hormodendrum* sp., the para form disappearing most quickly, while the ortho form was attacked very slowly. Three fungi, *Haplographium* sp., *Hormodendrum* sp., and *Penicillium* sp., were used for the chromatographical analyses, and in each case they produced the corresponding mono-hydroxybenzoic acid from the mono-methoxybenzoic acids (Fig. 4). Demethoxylation was also demonstrated in veratric acid (3:4-dimethoxybenzoic acid). From it vanillic acid was produced, i.e. the methoxyl group in the para position had been converted to a hydroxyl group.

Thus, it has been shown that soil micro-fungi are capable of demethoxylating and breaking down constituent units of lignin. However, this property could be of importance in the decomposition of lignin in soil only if these units can exist there in the free state.

Release of lignin-related molecules from wood sawdusts by fungi

Evidence that other fungi may release such acids was obtained by using *Polystictus versicolor*, a white rot (Henderson, 1955). When grown on a rich medium in the presence of spruce sawdust over a period of 6 months it released vanillic acid, which could be isolated on extraction of the sawdust with alkali. Similarly, on birch sawdust this fungus released vanillic and syringic acids. Soil micro-fungi were inactive in this respect. It is worth noting that the macro-fungus released from the softwood (spruce) and the hardwood (birch), the same units as are obtained on nitrobenzene oxidation of the lignins obtained from these woods. Since similar treatment of soils and peats has also been shown to release these units, it seems possible that, if there exist in soil basidiomycetes possessing enzyme systems similar to that present in the wood-rotting basidiomycete, lignin in the soil could similarly be broken down and its constituent units released.

Remarks on the tannic acid method as a means of detecting soil-fungi decomposing lignin

It was mentioned that the ability to colour tannic acid was used as a criterion in the isolation of fungi from soil samples. In the course of the experimental work, no evidence was obtained which indicated any correlation between the possession of enzymes which oxidize tannic acid and the ability to attack lignin-related compounds. This is not altogether surprising since the oxidation of phenolic compounds involves quinone formation and polymerization, while the attack on the phenolic compounds studied involved breaking of the ring, two entirely different processes. It, therefore, appears that the use of the tannic-acid method for isolating lignin-decomposing fungi has little foundation in the light of these results. After all, when lignin is removed by white rots from wood, the remaining product is lighter in colour than the original!

Conclusions

A surprising feature arising from the work is the widespread ability of soil micro-fungi to decompose aromatic compounds. The sixty fungi used in the preliminary survey with *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde, and vanillin belonged to some 30 different genera,

all of which exhibited some ability to decompose the compounds. Certain genera were more active than others and removed all traces of the four compounds from the media, while others could decompose completely only some of the compounds.

The approach to the problem of lignin decomposition which we are using at the Macaulay Institute gives, it is believed, for the first time one way in which lignin can be broken down under natural conditions by fungi. It is thought that it takes place by a primary release of the aromatic units of lignin, e.g. vanillin, etc., by macro-fungi followed by further breakdown of these compounds by soil micro-fungi. It is still not known how the basic units are released, but their further breakdown involves demethoxylation and hydroxylation followed by ring rupture, along paths probably leading into one of the common metabolic systems; for example, the Krebs Cycle.

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